

The importance of large animal models in transplantation

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1. ABSTRACT

Animal models have been extensively used in transplantation research. However, animal experimentation is contentious and subject to legal and ethical restrictions. Most experiments are carried out on rodents, but crucial prerequisites for the development of safe pre-clinical protocols in biomedical research are needed through suitable large animal models. In transplantation particularly, large animal models have developed dramatically. This article provides an overview of the large animal models commonly used to evaluate organ transplant experiments and analyzes the specificity of several models in various situations such as induction of allospecific tolerance and xenotransplantation. The key determination that remains to be addressed is the most appropriate species and strains to model human immune and physiological systems. Because of their phylogenetic and physiologic similarities to man, non-human primates play an increasingly important role in pre-clinical testing. Nevertheless, a number of studies have shown the pig to be a reliable large animal model for transplantation research, and the availability of genetically defined or modified pigs establishes a stronger position for pigs as a large animal model.

2. INTRODUCTION

Transplantation is today the treatment of choice for several end-stage organ diseases. Experimental research on large animal models has been critical for this phenomenal development in human medicine. Murray and co-workers (1,2) demonstrated in the early 1960s that a treatment with anti-metabolite drugs allowed to significantly prolong the survival of renal homografts in dogs: these experiments were crucial for the endorsement of transplantation as an overwhelming therapeutic tool. The remarkable results obtained in human transplantation, however, have led to a major worldwide shortage of organs and cells. This shortage is the impetus for research into novel methods to directly or indirectly increase the number of available organs and cells through tolerance induction, use of living-related grafts or non-heart beating donors, xenotransplantation, and cellular transplantation. Animal models will again play a major role in these research topics.

Although much knowledge has been accumulated about the immune systems of rodents and humans, the evolutionary gap between them has often hampered the

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direct applicability of the knowledge gained. Despite the advantages of using small animal models in transplantation, and the advantages conferred by congenic strains, transgenic, and knock-out murine models notwithstanding, recent data confirm that experiments in rodents are not sufficiently relevant to predict human responsiveness to potential treatment strategies. Extrapolating from rodent studies to human applications is difficult for several reasons:

- The young age of murine models certainly has an important impact on the maturity of the immune system and renders immunomodulation easier.
- The expression of major histocompatibility complex (MHC) antigens is restricted to antigen-presenting cells (3).
- The general conditions of rodents differ from those of large animals and humans. In fact, rodents have a reverse activity cycle compared with humans, so the chronobiology is different (4).
- The dosage/metabolism conversions between the rodents and NHPs/humans are always approximate (5).
- Different pharmacokinetics parameters might lead to incorrect extrapolation since chronological times are used instead of allometric ratios to determine the lifetime and the body weight of the rodents (6).

In contrast, large animal models, although complex and expensive, can be more relevant to clinical transplantation and to understanding the immunological barriers to transplantation. Large animal experimentation has a long history in the field of transplantation research, where it has been used from an anatomical and a surgical point of view to plan the technical aspects of the surgery, for research into physiological and immunological aspects to overcome the rejection processes, to determine the effectiveness of immunosuppressive agents, and to induce donor-specific tolerance. In this way, great strides have been made in recent years to develop reagents and tools to study immunology of non-human primates (NHPs) and pigs.

The aim of this article is to review the commonly used large animal models (pigs and NHPs) in the field of transplantation research, identify their advantages and limits, and propose guidelines for the use of animal models.

3. ETHICAL CONSIDERATIONS

The ethics of large animal experimentation have evolved to a level similar to that stated for humans: large animal experiments should be based on prior *in vitro* and, if possible, small animal experimentation. In each country of the European Union, animal research is currently controlled by national legislation. In Belgium, animal laboratory use is controlled by the Animal Law of 1986 and the European legislation—directive 86/609/EEC—for the protection of animals used for experimental purposes. These regulations aim to improve the controls on the use of laboratory

animals, set minimum standards for housing and care, standardize the training of staff who handle animals and supervise the experiments, and reduce the numbers of animals used for experiments. A research laboratory must hold a personal license authorizing it to perform regulated procedures on protected animals. This license authorizes specified work for a well-defined purpose and must include a detailed protocol for each regulated procedure. The protocol must be approved by the local animal research ethics committee of the institution maintaining the research laboratories, and the research can be carried out only under the supervision of an official animal care and welfare officer and a named veterinary surgeon for a clearly defined period of time.

Animal research in Belgium and in Europe is as such governed by the three “R” rules: reduce (lower) the number of animals used in experimentation, refine (improve) their life conditions, and replace laboratory animals if possible by *in vitro* or other experiments (7).

4. THE IMPORTANCE OF LARGE ANIMAL MODELS FOR TRANSPLANTATION RESEARCH

No known animal model can provide data that are completely comparable with humans, but most animal data provide a reasonable prediction of efficiency and safety. Animal models have played a critical role in establishing basic paradigms of immunology and physiology because they provide an *in vivo* milieu that cannot be reproduced *in vitro*. Insights gleaned into the mechanism of immune and physiological functions in rodents form the basis of research; however, an increasing knowledge of the complexity of the human immune system reveals important hurdles that do not appear in small animal models. Although numerous strategies have been successfully applied to small rodent models, extending most of these to large animals has been difficult.

In fact, *in vitro* studies cannot mimic the most basic *in vivo* immune responses because so many local and systemic cellular and humoral actors to auto, allo- or xenoantigens are involved. Animal models are thus an obvious step needed to identify the most relevant pathways involved in immune mechanisms that only can be hypothesized *in vitro*. These models nevertheless result from a stepwise progression that includes *in vitro* experiments in well-controlled systems, followed by the confirmation *in vivo*, in murine models. The need to pursue research in large animal models depends on the analysis of the data previously obtained. What exactly does the survival of a transplanted organ for 100 days in a young mouse mean, when it probably has a completely immature or naive immune system? Is it indefinite survival? Is it specific and indefinite tolerance? Thus, large animal models must serve as a confirmation step only when the data converge reasonably to suggest a possible application in human medicine. In addition, these data must have been reproduced in several conditions and laboratories before extensive additional experiments are considered.

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Table 1. Success of tolerance induction protocols in small versus large animal models and human

Method	Mouse	Primate/Human
Enhancement	+	-
Donor-Specific Transfusion	+	-
Peptides	+	-
Anti-MHC mAbs	+	-
Cyclosporin	+	-
Anti-Lymphocyte Serum	+	-
Anti-CD4 mAb	+	-
Anti-CD25 mAb	+	-
Total Lymphoid Irradiation	+	+/-
Anti-CD3 toxin	+	+/-
Co-stimulatory blockade	+	+/-
Chimerism	+	+

+: Tolerance induction success, -: Tolerance induction failure

5. ANIMAL MODELS

The first immune characteristic of small animal models is certainly the young age at the time of experimentation, which correlates with an immature immune system and a very active thymus. These young animals are usually inbred and very much protected from outside pathogens and immune challenges (viruses, vaccines, germs, etc.). This “artificial” environment is clearly different from an adult immune system, which has a large repertoire of T-cell memory. Also, the expression of class II antigens, which is in fact restricted to dendritic cells, could play a major role in small animal transplantation models: for instance, the lack of expression of class II antigens on vascular endothelium is related to the relative ease for inducing tolerance to primarily vascularized allografts in rodents, even across full MHC barriers (see Table 1) compared to NHPs or humans (8).

In contrast, large animal models are crucial, especially in transplantation immunobiological research, since class II expression of MHC antigens is constitutive on the endothelium as it is in humans. The importance of class II antigens has been extensively demonstrated in transplantation pig models and in NHPs that have undergone MHC antigen typing (9,10).

Among large animals dedicated to this research field, three models—dogs, pigs, and NHPs—have been and are still used frequently in transplantation research because their physiology and general anatomical and immunological properties approximate those of humans.

Large animal models were first used to develop the technical and surgical aspects of allotransplantation. In 1961, a surgical technique of heart allograft in dogs, which led to Barnard’s first human cardiac transplant in South Africa in 1967, was developed (11,12,13,14); similarly, liver allotransplantation was developed in pig and dog models to eventually allow the first human liver transplant (15,16,17,18,19). Calne and co-workers clearly initiated the first step of allotransplantation in humans by showing prolonged survival of renal allografts in beagles (1). Today, large animal models still allow technical development such as spleen allotransplantation to evaluate tolerance induction (20).

Dogs were abundantly used in early transplantation research. Their use in renal, heart (11,12,13,14), liver (18,19), lung (21), intestinal (22), bone (23), bone marrow (24), pancreas (25) and pancreatic islet transplantation (26) has been important for facilitating surgical training. In recent decades, the dog model has mainly been used in bone marrow transplantation, since the dog MHC antigen has been well characterized in strains such as beagles (dog leukocyte antigen [DLA]) (27). But over time, dogs have been less used in response to societal pressure. In addition, reagents for dog immunological studies are very limited and do not allow the study of immunological mechanisms. Most ethics committees mandate the use of breeding colonies for dogs. This renders their use more difficult and much more expensive. Dogs are still used to assess drug pharmacokinetics in transplantation.

The pig model is thus slowly replacing the dog model in many experimental protocols because this animal is accepted for human consumption. Thus, there is little public resentment of public research and its use is less expensive. Regarding the European legislation and in contrast to classical laboratory animals and domestic animals, outbred pigs for laboratory research may come from farms, and colony breeding is not mandatory. The pig has distinct advantages:

- It breeds very easily.
- Its anatomy, physiology, and immunology are well known and quite comparable to those of humans.
- It is reared and adapted easily to experimental conditions and has few disease problems.
- The ease of pig genetic engineering is a major advantage since the recent advances in biotechnology allow their transgenic breeding and cloning (28,29).

The pig is thus very important as an animal model and, as discussed later, is also a putative important source of cells and organs for humans. The use of pigs as a model has been paralleled by marked improvements in pig immunological markers such as monoclonal antibodies and pig cytokine detection (30).

The use of outbred pigs for transplantation immunology research is common, but the lack of MHC antigen control does not allow precise studies and consequently well-defined result analysis. For this reason mainly, Sachs D.H. developed his own inbred miniature pig model that allows researchers to control all the MHC combinations in several transplant models such as renal, cardiac, thymic, and bone marrow. In fact, inbred pigs with well-identified swine leukocyte antigen (SLA) (31) have been used, particularly in allotransplant research (10). This miniature pig model mimics all human MHC combinations (10) and has a slow growing curve, but has one major disadvantage related to breeding difficulties: small litters caused by significant consanguinity. Certain minipig breeds (Göttingen minipigs, Yucatan pigs) are also used,

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particularly for biomedical research because of their reasonable size (adult weight = 30 kg).

Pigs need to be used at a reasonable weight to perform transplantation surgery in laboratories with sterile conditions, so the age of the animals is mostly approximate to very young adults rather than to adults. As in rodents, the immune systems of young pigs are still immature, and the memory phenotype is still developing and the peripheral T cell repertoire is predominantly naive. In addition, these young animals have still a very active thymus at 3 to 6 months of age, which must be taken into consideration for transplantation research. The data with experiments in adult pigs should therefore still be confirmed when results are found in young pigs. For this reason, the use of miniature pigs from inbred colonies is a considerable advantage since adult pigs can still be used at 150 kg, whereas outbred pigs of similar age have often a weight of 250 to 300 kg, which precludes their use in the laboratory.

NHPs have several important properties that allow them to serve as a bridge between the basic insights obtained in other animal models and their application to humans (32). According to the Convention on International Trade in Endangered Species of wild fauna and flora (www.cites.org/eng/disc/text.shtml), experimental research with *Hominidae* (the great apes) is prohibited except for research in virology (AIDS and hepatitis). The two *Cercopithecidae* families (baboons and macaques (rhesus and cynomolgus) from the Old World provide most of the monkeys used for biomedical research, especially in hematology, immunology, and virology (33).

The primate immune system has important similarities to humans:

- A high level of homology has been reported between humans and baboons for MHC class I molecules (90%) (34).
- Nucleotide and protein sequences of several cytokines are almost homologous in humans and macaques (93% to 99%) (35).
- The similarities between the sequences of macaque and human antibodies are equivalent to those between human antibodies that originate from different individuals (36).
- The ABO system, hemoglobin, coagulation and fibrinolysis physiology are comparable between humans and baboons (37,38).

Although the immune system of NHPs has important homology to the human immune system, there are some notable exceptions such as antibodies for *Macacus* CD3, which do not cross-react with human CD3 molecules and vice versa. In contrast, many CD3 molecules are still unknown in *Macacus* in comparison to human molecules and the determination of immunoglobulin subtypes is still imprecise in baboons (39).

The use of NHPs however, faces considerable hurdles, including social pressures, pathogen-free animals (viruses), major expense, complexity of care, and low rate of fecundity, but they represent crucial models for

transplantation research, especially in the field of tolerance induction. Their size could present a problem because of practical surgical aspects, but allows easier drug dosing. Adult baboons weigh 20 to 25 kg, which allows repetitive blood sampling and represents a good model compared with pig organs in a xenotransplantation context. But because there are no breeding colonies, the availability of this species is limited. The most used NHP models in allo- and xenotransplantation are today the cynomolgus and rhesus macaques, as colonies live in various countries and delivery is possible by following the rules in force.

The importance of using NHPs for biomedical research has been emphasized by the European Union. In the absence of *in vitro* models that can take into account the complexity of humans, the use of NHPs remains the gold standard before clinical trials. As will be described later, the NHP model is the only real model to allow comprehensive studies in xenotransplantation.

6. FACTORS CONTROLLING ALLOGRAFT OR XENOGRAFT REJECTION

6.1. Allotransplantation

The rejection of human allografts may be mimicked by large animal models, and immune responses are mostly dependent on similar immune T-cells that respond to the major histocompatibility antigens. However, minor antigens, blood group and recipient status are also involved:

- MHC antigens: these are of overwhelming importance in determining the outcome of allotransplantation in large animal models and in humans. The matching of class II antigens has been particularly emphasized in human and pig models (10). In hyperacute and accelerated allograft rejection, sensitized recipients may reject their allografts within minutes or hours, through pre-existing anti-MHC antibodies against the donor. Models for this type of rejection include pig models in which the recipient is previously sensitized by skin grafts from the future donor. This hyperimmunized pig model is of overwhelming importance to study the control of anti-MHC antibodies elicited in humans caused by previous grafts, blood transfusions or pregnancies (40).
- Non-MHC linked immune response and minor antigens may play a part in allograft rejection in several animal models.
- Blood group antigens may also produce hyperacute rejection (HAR) and need to be controlled in all large animal models. Although blood group is easily controlled in inbred pig models, outbred herds show ABO incompatibilities and 16 porcine blood groups are recognized (41). These ABO antigens must also be matched to avoid unexpected conclusions; e.g., humoral rejection, on data generated by such models. The same has to be done in NHP models, at least in allotransplantation.

6.2. Xenotransplantation

In xenografts, less is known about cellular rejection in discordant xenogeneic grafts, but enough data seem to evidence the similarity of the humoral immune

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response to xenoantigens to consider the pig-to-primate model as the only model that is appropriate for predicting data in humans.

Xenotransplantation is divided into two types, concordant and discordant, according to the discrepancy in species. Concordant xenotransplantation is a transplantation across species in which there are no serum preformed antibodies; e.g., chimpanzee-to-human or chimpanzee-to-baboon. Discordant xenotransplantation is a xenograft across more distant species in which the recipient possesses circulating preformed antibodies against the donor antigens, e.g. pig to primate or pig to human. Humans, apes and old-world monkeys (baboon, *Macacus*) have antibodies against Gal- α (1,3)-Galactosyl determinants, which are widely expressed on pig vascular endothelial cells. These xeno-antibodies, which are comparable to ABO isoagglutinins in humans, probably result from sensitization to enterobacteria that rapidly colonize the small bowel after birth (42). These pre-existing (or xenoreactive) natural antibodies (XNAs), mainly of IgM isotypes, bind to α -Gal epitopes, activate the classical complement cascade, attract platelets, induce thrombus formation and activate the coagulation pathways that lead to rapid interruption of the graft function within seconds or minutes (43). This phenomenon is called hyperacute rejection. After overcoming this rejection process by either eliminating the XNA reaction or by blocking the complement activation, the organ graft can undergo acute vascular rejection that is characterized by antibodies elicited against Gal residues, infiltration of monocytes and natural killer (NK) cells, interstitial hemorrhage and intravascular coagulation. Even if these two types of vascular rejection are controlled, the xeno-organ can still be rejected by cellular mediated rejection in which NK cells and T-lymphocytes are involved (44,45,46). So far, chronic rejection in xenotransplantation has not been really studied in large animal models because no model survived enough long to evaluate this type of immune response.

The problems with vascularized organs need more research in large animal models, but xenogeneic cell transplantation might be less invasive and cause less severe immune reaction, as will be discussed later.

7. IMMUNOSUPPRESSIVE DRUGS

Humans and large animal models show generally similar physiological responses to most drugs tested if their delivery is adequately controlled. The main differences between large animals and humans are, however, the consequence of drug metabolism or absorption and lead to a drug response that is difficult to predict. In general, the only way to use drugs in large animals is to refer to whole blood or plasma levels and not to the dose given. For instance, to reach a therapeutic level in pigs or rodents, calcineurin inhibitors must be used at significantly higher doses than in humans (47,48), but this higher dosage does not preclude lower level use in humans if the circulating levels are comparable. In pigs, an increased volume of distribution and clearance, as well as a diminished systemic availability, explain lower plasma concentrations of cyclosporin A and prednisolone compared to humans. Thus, to obtain the same target concentrations of CyA

and prednisolone in both species, pigs require about 2 to 4 times higher I.V. or oral doses of CyA and 10 to 30 times higher I.V. or oral doses of steroids (49).

As a consequence, all the experiments done in pigs with a high dosage of calcineurin inhibitors should not be discarded as nonapplicable to humans, but should be evaluated carefully. In the early era of cyclosporin A, the dosages used in humans were much too high. Unfortunately, some renal allografts were lost because of the high dosages and consequent nephrotoxicity. Nevertheless, each situation must be clearly analyzed because stable tolerance has been extensively demonstrated in miniature swine with a regimen of 12 days of CyA at 10mg/kg/day. These results could be categorized as inapplicable to humans because the dosage is too high, but adaptation to the pharmacology in pigs would allow researchers to compare the effect with a two- to fourfold reduction in other mammalian species such as humans. The truth is that very few physicians would consider using only one simple drug to induce tolerance in humans, but several clinical trials recently demonstrated very good results in liver transplantation with a regimen that included only tacrolimus (50). In addition, true tolerance has been induced in inbred swine for completely mismatched renal allografts with clinically applicable low doses of tacrolimus (0.1 mg/kg/day: 7 to 20 ng/ml level in the serum) as well as liver tolerance with 0.1mg/kg/d for 12 days in semi-identical pigs (51,52).

In total, cyclosporin A and tacrolimus have been evaluated in dogs, pigs and NHPs with reproducible prolongation of graft survival, or even tolerance. Azathioprine and mycophenolate mofetil have been extensively tested in dogs, whereas sirolimus was not well tolerated by dogs or NHPs. In fact, the poor absorption of sirolimus by NHPs has led to the use of increasing quantities of the drug and consequent bowel lesions. Finally, sirolimus has mainly been evaluated in monkeys at low doses and in the context of multiple drug therapies (53,54).

8. IMMUNOSUPPRESSIVE THERAPIES IN LARGE ANIMAL MODELS

8.1. Monoclonal antibody or fusion protein therapy

The main problem with monoclonal antibody therapy in large animals is obviously the specificity. Therefore, many monoclonal antibodies have been tested in NHPs but very few in other large animal models because there is little or no cross-reaction (55,56). New monoclonal antibodies have emerged that may be viable candidates for use in innovative low-dose drug-sparing immunosuppressive combinations. With the high degree of human specificity, many mAbs used in animal models have been shown to be relevant in clinical human transplantation and identified to target immune activation, except CD3 whose human-CD3-specific agents generally do not cross-react with rhesus macaques (57).

Blocking the two main co-stimulatory targets (B7-CD28 and CD40-CD154) has been the main objective and blocking these two receptors synergistically induced tolerance in mice (58,59). In NHPs, monoclonal antibodies

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specific for CD80 and CD86 have significantly prolonged the time of renal allograft survival when used simultaneously but have not resulted in tolerance. The injection of either anti-CD80 or anti-CD86 only moderately prolonged renal allograft survival (54).

Injection of CTLA-4-Ig, the alternate soluble protein that binds CD80/86, provided limited efficacy in single use in *Macacus* (60). A new mutant version of CTLA4-Ig (with a better affinity for CD86) and a combination of rapamycin and anti-IL-2-receptor antibody, significantly improved graft acceptance in rhesus monkeys (61). These results seem to be confirmed in recent human studies (62).

Monoclonal antibodies directed to CD40 or CD154 (CD-40L) have been used in *Macacus mulata* and *M. fascicularis*. These mAbs allowed to demonstrate very prolonged allograft acceptance of kidneys, hearts and islets (63,64,65). However, the use of one of this anti-CD154 mAb—hu5C8—in human renal transplantation elicited thrombotic side effects that were also observed lately in NHPs (66), and thus precluded continued clinical trials. Anti-CD40 mAbs were similarly tested in the same species and prolonged the survival of islet and kidney allografts; however, long-term acceptance and true tolerance were not observed (67,68,69).

Several researchers have tried to combine either anti-CD80/86 antibodies or CTLA-4-Ig with anti-CD154 in a search for synergistic effects, as observed in mice. A monoclonal antibody specific for CD154 resulted in markedly prolonged survival of kidney, islet, cardiac, and skin allografts, but, again, most animals eventually developed rejection after prolonged periods of rejection-free survival off therapy (63,64,65,70,71). Two renal-transplanted cynomolgus monkeys that were treated with anti-CD86, anti-CD40 mAbs and a short dose of cyclosporine, showed a very long survival (43 months) of the graft before chronic rejection. The combination of these targets gave better results than either treatment separately by prolonging graft survival (72).

Anti-IL2 receptor antibodies have been tested in rhesus monkeys before clinical use: Daclizumab (humanized mAb) and basiliximab (human/mouse chimeric mAb) have been thus successfully introduced into clinical use in association with calcineurin inhibitors, corticosteroids and mycophenolate mofetil (73,74).

The anti-CD20 mAb (rituximab, rituxan, mabthera) is active on all cells in B-cell lines except pro- and plasma cells from cynomolgus monkeys. These mAbs have been used to study new agents aimed at B-cell depletion in several human diseases (75).

8.2. Depletion strategies

Other therapeutic approaches involving monoclonal and polyclonal antibodies have been extensively evaluated in large animal models (76,77).

Several anti-thymocyte globulins (ATGs) were used in dogs, NHPs, and pigs, but none was tolerogenic, although long-term survival was occasionally observed (78). Rabbit anti-human thymocyte globulin has been especially studied in NHPs, but the dose needed to achieve complete depletion in NHPs is larger than that used in humans and the depletion was lower than in humans. This was probably the consequence of a lack of specificity. RATG was then used as an add-on treatment to total lymphoid irradiation or Cyclosporin A to increase T-cell depletion (79).

To achieve T-cell depletion in NHPs, however, the most impressive results have been obtained in rhesus monkeys with a monoclonal antibody directed against the CD3 molecule and bound to a modified diphtheria toxin (80). This treatment achieved total T-cell depletion, which allowed a significant prolongation of renal graft survival in rhesus macaques with induction of functional tolerance in 30% of the animals. This therapy provoked dramatic T-cell depletion within hours of the first injection and the repopulation of T cells could take up to 6 months. Although initially nonspecific, this treatment could induce acceptance of specific donor skin grafts and suppression of MLR and CTL production (81). Associated with immunosuppressive drug therapy such as deoxyspergualin, steroids and aspirin, this antibody showed the most impressive data in monkeys by producing survival longer than 2 years with some longer than 5 years (82). This therapy also has been successfully used in an islet allograft model with insulin independence over 1 year (83). The probable toxicity of the diphtheria toxin, however, has so far precluded any human clinical trials. The main effect of this drug is certainly to cause strong peripheral T-cell depletion, but in addition, T-cells from the lymphoid compartment were also rapidly killed. Such an effect has never been achieved with another antibody therapy and only TLI or TBI could provide such lymphoid depletion.

Anti-CD52 rat IgM mAb (also known as campath-1 [alemtuzumab]) similarly causes profound T-lymphocyte depletion associated with consumption of complement in cynomolgus monkeys and has been tested in human trials in which “prope” tolerance has been induced (84). In this case, however, the results in animal models were mild, whereas they seem very attractive in human trials. This is an example in which the lack of benefit in animal model could have led to the failure to develop this useful antibody.

Anti-primate CD2, LO-CD2b produces strong depletion of all peripheral CD2+ cells, including NK CD2+ cells, and represents an important immunological tool that can be used in preclinical models in baboons or cynomolgus monkeys. (85,86). In fact, the corresponding rat anti-human CD2 (LO-CD2a/BTI322) has also been successfully used in liver transplant patients (87), but did not significantly decrease the incidence of acute rejection episodes. The humanized form LO-CD2a (MEDI-507) has been tested in psoriasis and now in some lymphomas under the name of siplizumab.

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Table 2. Comparison of rejection process in xenogeneic animal models

	Small models		Large models	
Animal model	Guinea pig to rat	Rat to GalT-KO mouse	Baboon to monkey	Pig to monkey
Hyperacute Rejection (HAR)	HAR Alternative pathway/ controversial role of xenoantibodies	HAR T-cell dependent rejection	NO HAR	HAR Classical pathway
Animal model	Hamster-to-rat	Mouse-to-rat		
Acute Vascular Rejection (AVR)	AVR T-cell independent, B-cell role unclear	AVR MHC independent process	AVR Humoral rejection	AVR Vigorous humoral response
Animal model	Hamster-to-rat		Pig-to-NHP	
Accommodation	YES		NO	

Total lymphoid irradiation (TLI) targets the transient elimination of recipient T-cells that create a window during which a tolerance protocol might be used for allografts. TLI is as such used in addition to other immunosuppressive regimens to induce mixed chimerism and thereby central tolerance in monkeys (88,89). A comparable protocol that includes thymus-specific irradiation, bone marrow transplantation, ATG or MEDI-507 and cyclophosphamide was thus designed in humans to induce central tolerance in patients suffering from multiple myeloma. These are the first intentional and successful cases of clinical tolerance, which was clearly tested and evaluated similarly in primates by the same team (90).

8.3. Mixed chimerism

Following the notable success of mixed-chimerism approaches to induce tolerance in murine models with permanent acceptance of skin grafts across full MHC barriers, similar strategies have been applied successfully in NHP models. Donor bone marrow cell transplantation was infused after host T-cell depletion with TLI, ATG and specific high irradiation of the thymus in monkeys: when lympho-hematopoietic chimerism in the peripheral blood reached higher than 1% for at least 1 month, donor-specific tolerance to renal allograft was achieved (88,89,91,92).

8.4. Donor antigen infusion

Signal intensification by additional donor antigen infusion such as donor whole blood, lymphocyte or bone marrow infusion in combination with T-cell depletion and either co-stimulation or signaling blockade (anti-CD154 mainly) has led to successful tolerance induction in rodents, in allotransplantation and even in rat-to-mice transplantation (93). In NHPs, no prolongation of kidney allograft survival by combining donor-specific transfusion (DST) with anti-CD154 treatment was observed (69).

9. LARGE ANIMAL MODELS IN XENOTRANSPLANTATION

Xenotransplantation is a worthy example of the difficulty of choosing an appropriate animal model. Depending on the discrepancy between species involved, concordant and discordant models have been described. Several experimental models have been extensively developed in small and large animal models to mimic these situations.

The four rodent species commonly used in xenotransplantation research are the rat, mouse, hamster and guinea pig. The classical discordant rodent model used to study HAR has been the guinea pig-to-rat heterotopic heart

transplant model. But the role of xenoantibodies in this model was controversial and Pruitt *et al* have suggested that complement activation via alternative pathways plays a major role in rendering this model questionable (94). In addition, xenograft survival has not been prolonged over 1 or 2 weeks in this model (95). This model has therefore been abandoned.

The rat-to-mouse or hamster-to-rat models have been extensively used to study the xenogeneic immune reaction, but these models are closer to concordant models, although a few preformed antibodies might be evidenced. The hamster-to-rat model is, however, interesting to study since the process of rejection is mainly T-independent and the activity of the B cell compartment and the NK activity have been studied successfully in this model (96). In addition, accommodation has been described in the hamster-to-rat model, and has enabled several important protective genes to be discovered that could play a major role in accommodation induction (97).

Because the pig has been identified as the likely donor for human cells and organs, and the Galactosyl residue has been identified as the main target of human and primate preformed antibodies, Gal-knockout mice have been produced to mimic that situation. This model is interesting for studying the anti-Gal response, but in these animals HAR is T-cell dependent and alpha-Gal expression in mouse tissue is 200 to 300 times lower than that in pigs, which demonstrates the limits of this small animal model (see Table 2) (98).

9.1. Concordant large animal models

Historical trials of animal organs being transplanted into patients have been reported, but cases with significant clinical results are rare. Until the mid 1960s, primate organs were transplanted to humans. In 1964, the most significant clinical success in xenotransplantation was obtained by Reemtsma, who transplanted kidneys from chimpanzees into humans. The longest survival was 9 months, which represented an incredible result at that time with the very limited immunosuppressive drugs available. No sign of rejection was observed at autopsy, which suggested that the patient died from a water-electrolyte imbalance. Six additional patients were given baboon kidneys and the concordant xenografts functioned properly for up to 60 days, but the doses of azathioprine and prednisone required were high, and the grafts were eventually rejected (99). In 1985 Bailey successfully transplanted a baboon's heart into baby Fae, who was born with a congenital heart anomaly. She survived for 20 days, and the loss of the xenograft was probably related to ABO blood type incompatibility (100).

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Primates from the *Hominidae* and *Cercopithecidae* families are immunologically concordant with humans. Based on the patient survival rates, primates, especially chimpanzees, appear to be the most suitable xenograft donors. However, these primates are today threatened with extinction and are difficult to breed. The very narrow proximity in the phylogenetic tree creates important ethical issues against the use of these animals in experimental protocols. Moreover, chimpanzees could be a source of dangerous pathogens, including Ebola and human immunodeficiency virus.

Other large animal concordant models include baboon-to-monkey (*Papio anubis* and *Macacus fascicularis*) and wolf/fox/dingo-to-dog models. The combination baboon-to-macaque or vice-versa was used to investigate immunosuppressive protocols for optimal clinical xenotransplantation. Untreated recipients survived 6 days, whereas monkeys with cardiac or renal xenografts under immunosuppressive drugs survived 149 to 380 days (43). With concerns about zoonosis, viral recombination, and other ethical issues, NHPs are *not currently considered feasible and safe donors for humans*.

9.2. Discordant large animal models

The pig has been identified as the animal most likely to provide donor organs on a large scale for transplantation into humans. Pigs do not suffer social or ethical pressure, germ-free animals can be reared, their physiopathology is well known and documented, and last but not least, pigs grow rapidly and their genetics are well known. The possibilities of producing transgenic pigs and most usefully and of knocking out genes and cloning pigs are major arguments today to justify their use as putative cell and organ donors for humans.

The immediate consequence of choosing a discordant model for xenotransplantation also has a direct impact on large animal models. In fact, the main residue, which is recognized by preformed antibodies from humans and primates on the pig endothelium, is galactosyl (101); therefore, we must pursue the research in this field with a model that can mimic that situation. The direct relevance of other animal models, such as concordant models, or discordant models such as pig-to-dog, monkey-to-pig, or ratite (emu, ostrich)-to-baboon, to clinical XT_x in humans is thus questionable. The different antibody-antigen systems involved and the possibility that complement activation occurs via an alternative pathway as well as, or instead of, the classical pathway, render these models without real interest.

The pig-to-primate model (either macaques or baboons) is therefore the only true model for mimicking the hypothetical situation of pig-to-human cellular/organ xenografts. This does not mean that there is no place for mechanistic immunology using small rodent models such as hamster-to-rat or rat-to-Gal-KO mice. In fact, these models need to open the ways to discovering new molecules or genes that may be involved in xenograft rejection.

9.3. The prevention of discordant xenograft hyperacute rejection

As previously described, the first immunological hurdle in the pig-to-primate or human model is HAR. Several major approaches have been explored in pig-to-primate experimental models to overcome HAR: (1) depletion or inhibition (or “neutralization”) of anti- α Gal antibodies (102), (2) depletion or inhibition of complement (103,104,105), and (3) the use of organs from genetically engineered pigs that are transgenic for human complement-regulatory proteins (106,107). The two first therapeutic approaches allow researchers to prolong xenograft survival up to several weeks instead hours, but transgenic organs have provided the most significant results.

9.3.1. Transplantation of transgenic pig organs

Transgenesis appears to be an essential tool to study rejection mechanisms and a potential way to engineer pigs to be used as organ donors for humans (108). So an alternative and important plan has thus been to produce transgenic pigs that overexpress human complement regulatory proteins (CRPs) such as hDAF/CD55 (decay-accelerating factor), hCD59 (membrane attack complex) and hCD56 (membrane cofactor protein). Organs from these animals are protected from HAR because they express CRPs that are compatible with the recipients (106,107).

The combination of using transgenic pig organs and depleting anti- α Gal antibodies has produced interesting results. Kidneys from these transgenic pigs have functioned for 2 to 12 weeks in monkeys treated with conventional immunosuppression, whereas control pig kidneys were destroyed after a few days (109,110,111).

Improved graft survival using hDAF pig hearts and blocking anti-Gal antibodies by infusing α Gal glycoconjugate and a human anti-human CD154 mAb improved graft survival by an average of 37 days (4 to 139 days) (112). Cardiac xenografts in baboons using hCD46 associated with a heavily immunosuppressive regimen (α Gal polymer, rituximab, tacrolimus, sirolimus, steroids, rabbit ATG, and splenectomy) improved graft survival time to 56 to 113 days (mean 76 days), but viral infection (baboon cytomegalovirus) emerged as a serious problem (113).

Again, the large animal model is of overwhelming importance because, for instance, cynomolgus monkeys seemed more tolerant to hCD55-pig organs than were baboons. Therefore, the use of the two NHPs models seems crucial to confirm data before human studies can be conducted (114).

The data obtained with several lines of transgenic pigs that express one, two or three human regulators of complementary genes certainly enabled progress in the discordant pig-to-primate model. The main breakthrough was the control of HAR and the extension of graft survival to 3 to 4 months with such transplants. One major problem, however, remains crucial: acute vascular rejection

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(AVR), which occurs during the weeks after xenograft and despite HAR being controlled. This vascular rejection depends on preformed and elicited anti-pig antibodies and on innate immune cells such as polymorphonuclear cells, NK cells, macrophages, and later T-cells. In addition, this vascular rejection is correlated with several problems such as acute systemic thrombocytopenia and consumptive coagulopathy in association with localized intravascular thrombosis. These abnormalities could be related to the disparity of hemostasis pathways between discordant species. Some of these coagulation disorders and platelet physiology incompatibilities were inhibited by heparin derivatives, antithrombin III, and proctacyclin inhibitors (115). The only real solution to overcome several of these problems together was obviously to consider knocking out the expression of the galactosyl residue on pig endothelium. This is the third main step that has been taken during recent years in the field.

9.3.2 .Alpha-1, 3-galactosyltransferase knock-out pigs

The first homozygous GalT-KO pigs were developed in 2003 by knocking out the galactosyl transferase genes in early pig cell embryos (29,115,116). The production of these pigs demonstrated that survival times surpassed previous attempts using donor organs from non-GalT-KO pigs (16 to 56 days). Kuwaki *et al.* transplanted GalT-KO heterotopic pig hearts into baboons and the xenografts ceased contracting on days 59, 67, 78, 110 and 179, the latter being the longest survival recorded in any porcine-to-NHP model to date (117).

The survival of pig “thymo”-renal transplants from GalT-KO pigs has been reported in cynomolgus monkeys undergoing a tolerance induction regimen. Survival was prolonged up to day 81 to 83 after transplantation (118).

The use of Gal-KO pigs, however, also evidenced that the next hurdle for discordant xenotransplantation will be non-Gal antibodies as well as the development of thrombotic microangiopathy. The causes of these hurdles probably involve activation of the vascular endothelium by non-Gal antibodies as well as innate immune cells which recognize non-Gal antigens, and coagulation dysregulation caused by molecular incompatibilities between pig and primate (115). As discussed in perspectives, GalT-KO pigs that express human complement-regulatory protein such as CD55, plus anti-coagulant genes such as TFPI and thrombomodulin, will be necessary to overcome these problems and go a step further.

9.4. Cell-mediated and chronic rejection

After HAR and AVR have been controlled, the xenograft undergoes cell-mediated rejection involving NK cells and T lymphocytes. The NK cells exhibit cytotoxicity to vascular endothelial cells, whereas the T lymphocytes respond to xenoantigens presented directly or indirectly and induce a more severe cell-mediated rejection than that observed in allotransplantation (119). The role of T cells in xenograft rejection is not yet well understood, but there are probably important mediators in cell-mediated rejection which will need classical immunosuppressive agents at

higher doses if they are to be controlled. A beneficial situation, however, with pig-to-human xenografts, would be if genetic manipulation of the ideal donor could be pursued and genetic engineering of the T-cell response such as expression of CTLA4-Ig on pig cells could also be carried out.

Until now, chronic rejection in xenotransplantation has been unknown, but if a pig organ is successfully accepted long term, chronic rejection is likely to occur.

9.5. Accommodation

Accommodation is a phenomenon by which vascular endothelial cells show resistance to an antibody-mediated injury. Accommodation was first demonstrated in ABO-incompatible human living-related renal allografts and then used as a model for the experimental discordant xenograft model (102,120,121). The mechanisms of accommodation have been relatively well studied in hamster-to-rat heart xenotransplantation, but never in large animal models. Protective genes such as anti-apoptotic genes A20, bcl-2, bcl-X_L and others including genes that code for hemoxygenase-1, have been extensively studied and identified as being involved in this phenomenon (97). Accommodation will certainly be studied again in pigs that have been genetically manipulated (97).

9.6. Induction of immunologic tolerance

A protocol including whole body and thymic irradiation, splenectomy, a course of antithymocyte globulin, and cyclosporin, together with an allospecific bone marrow infusion, resulted in tolerance to renal allografts in cynomolgus monkeys, with graft survival of longer than 3 years in the absence of any form of therapy after the first month (63). This regimen, however, is not yet adequate for tolerance to pig organs, even when combined with antibody immunoadsorption (pig liver perfusion or immunoaffinity columns of synthetic alpha-Gal). The return of xenoantibodies systematically resulted in the rejection of pig organs in cynomolgus monkey and baboon recipients. When a donor pig thymus was transplanted together with the pig kidney (“thymo-kidney”) into a baboon that was pretreated with whole body irradiation and immunosuppressive therapy, xenograft survival was extended up to 3 months but without tolerance (118).

10. NON-IMMUNOLOGIC HURDLES

10.1. Anatomical and physiological obstacles

Regarding compatibilities, data show physiological compatibilities between pigs and primates, e.g. coagulation factor VII. Two baboons were reported to have tolerated a liver xenograft for 4 to 8 days with normal oral intake, normal coagulation tests, and porcine fibrinogen (122,123). In primates with prolonged posttransplant survival, renal function has been preserved before rejection, as shown by essentially normal serum creatinine, chloride, potassium, and calcium levels, providing that a kidney from a phylogenetically distant species can maintain fluid balance and normal biochemical homeostasis in primates. Nevertheless, despite the

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administration of recombinant erythropoietin, NHP recipients suffered from severe anemia (107).

There are also several incompatibilities, e.g., complement cascade, protein C and thrombomodulin. Blood viscosity, enzymes and hormones show some variations between humans and pigs (124). Coagulation disturbances are encountered in discordant models. These abnormalities are related to the disparity of hemostasis pathways between discordant species. Acute systemic thrombocytopenia and consumptive coagulopathy, in addition to localized intravascular thrombosis during AVR, create another barrier. Models using hearts from transgenic mice that express anticoagulant fusion proteins showed graft survival prolongation up to 100 days in Lewis rats, whereas control hearts were rejected within 3 days (125). This novel strategy must be considered in pigs. *In vitro* studies showed that recombinant porcine fgl2, a prothrombinase, could generate thrombin from human prothrombin. *In vivo* pig-to-baboon xenografts undergoing AVR were positive for inducible pfgl2 on endothelial cells. These incompatibilities between pigs and humans need further consideration (126).

Besides immune hurdles, anatomical (size, posture) and, especially, physiological differences exist between humans and pigs, but these characteristics will only be available for study when reproducible and long-term survival of a pig organ into a primate has been achieved (110,127).

10.2. Zoonosis

Pigs have pathogens, bacteria, fungi, parasites, and viruses, but they are preferable to NHPs as a source of donor organs because recombination could be less dangerous or could occur less frequently than with primates, which are close phylogenetically. A major concern, however, is the potential for xenogeneic transmission of viruses from pigs to humans via organ, tissue, and cellular transplantations or via *ex vivo* exposure of humans to porcine biologic materials. Immunosuppressive drugs may facilitate the transmission of zoonotic agents. Viruses that cause apparent diseases in pigs are relatively easy to eliminate from donor herds, but those that are asymptomatic in pigs and those that undergo latency are more difficult to eliminate. Among them, porcine endogenous retroviruses (PERV) are of particular concern. Three subtypes of PERV exist in germ line DNA and are human-tropic replication-competent; so they are relevant to clinical xenotransplantation (128). These viruses may form in the recipient posttransplant recombination and lead to a new virus human-competent. All *in vitro* and *in vivo* studies to date have shown the absence of PERV transmission in several hundred patients tested, which is encouraging (129). Continuous research with *in vivo* models is, however, required to draw clinically significant conclusions and to determine whether zoonosis will be a manageable component. Pig strains that express no or fewer retroviral sequences have been found, but PERV-KO pigs could be engineered in the future (130).

11. EXPERIMENTAL CELLULAR XENOTRANSPLANTATION

Over the last decade, experimental and clinical assays have generated exciting data on the role of cellular transplantation (islets, hepatocytes, retinal cells or brain cells) as treatments for insulin-dependent diabetes mellitus, acute liver failure, retinal degeneration, and Parkinson's disease, respectively. Among the xenogeneic cellular models under investigation, islet xenotransplantation is the most active. In fact, using pigs as islet donors would offer an unlimited number of islets. Recently, two main studies demonstrated that a heavy immunosuppressive regimen that includes, in particular, anti-CD154 mAbs, could control diabetes in STZ-treated or pancreatectomized primates up to 6 months (131,132). These two main studies will certainly provoke a resurgence of interest in xenogeneic islet transplantation, once several additional studies have been done to render the immunosuppressive regimen more applicable to humans. In addition, recent data obtained in our laboratory tend to demonstrate that immuno-isolation by alginate encapsulation of pig islets can correct STZ-induced diabetes in monkeys of up to 6 months without any immunosuppression. These results were obtained while there was an antibody response against porcine antigens in the serum, but the encapsulated pig islets were not affected by this humoral reaction (133).

Hepatocyte transplantation is still innovative therapy in allotransplantation (134), but xenotransplantation of hepatocytes has already been studied in small animal models; for instance, a significant decrease in serum cholesterol has been shown in Watanabe rabbits after transplantation of healthy porcine hepatocytes (135). Similarly, clinical parameters were significantly improved in cirrhotic rats after transplantation of porcine hepatocytes (136). Immunodeficient mice have been used as xenotransplantation models to develop *in vivo* models of hepatitis B or C virus infection. These data show that human hepatocytes can repopulate the mouse liver and indicate no fundamental incompatibility between the murine liver microenvironment and human hepatocytes (137). These results demonstrate the feasibility of this approach to support liver failure by xenogeneic cells, but they need to be validated in preclinical large animal models. A recent work showed interesting data in the pig-to-primate model with hepatocytes survival for 80 days after the first injection and 253 days after a second injection (138).

In humans, bioartificial liver devices containing porcine hepatocytes have been extensively studied in patients and serve as a bridge to liver allotransplantation in the case of fulminant hepatitis (139). Bioartificial liver support based on porcine hepatocyte system was first developed in a canine model (140).

Clinical cell transplant trials have been performed in patients with Parkinson's disease, Huntington's disease, demyelinating diseases, retinal disorders, stroke, epilepsy, and even deafness, and normally are designed as cell replacement strategies. Major practical and ethical

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problems with the use of aborted human tissue in clinical transplantation programs prevent its widespread adoption. This has led to the search for alternative sources of cells, including some from other species, such as the pig. There are 200,000 dopaminergic neuroblasts in the pig, and the optimal age for harvesting these cells appears to be embryonic days 26 to 27. If cells are taken at this age and xenografted into the adult central nervous system, they are rejected through a combination of cellular and humoral immune processes. The immune responses induced against this discordant embryonic porcine tissue have been addressed in mice and rats, and the critical factors for graft rejection have been determined (141). Conventional immunosuppressive drugs used as monotherapy (e.g., cyclosporin A or tacrolimus) do not protect grafts (142). Short courses of treatment with molecules that block T-cell co-stimulation (CD40L, LFA1 and CTLA4Ig) have resulted in very good graft survival in mice (67), although long-term studies are needed to determine whether this therapy could be used clinically. Transgenic porcine VM tissue, expressing either the human complement inhibitor CD59 or human α -1,2-fucosyltransferase in combination with a recipient treatment with anti-C5 antibodies (H transferase), survives up to 12 weeks in Parkinsonian primates (143). However, the level of expression of these human complement-regulatory proteins is generally low in transgenic embryonic pig brains.

12. PERSPECTIVES

Rapid progress in biomedical research has generated a huge number of experimental data from mostly small animal models, but there is little doubt that large animal models have provided an invaluable insight into transplantation research.

Small and large animal models have advantages and disadvantages, but guidelines need to be followed to take into account new ethical rules, obvious social pressures, scientific progress, and appropriate use of animal models.

Considering that transplantation medicine today faces a major organ shortage and that over-immunosuppression is totally unacceptable, the new challenges for transplantation are related to increasing the organ pool and improving the specificity of immunosuppression.

To increase the number of available organs, the whole network of organ harvesting must be continuously improved, which is clearly ongoing. Optimizing the use of living-related donors is also ongoing in all transplant centers, but the main problem will be to protect society against any dysfunction such as sales of organs at this level. To increase the pool of available organs, the use of non-heart beating donors is and will continue to be a major field of improvement. In this area, experimental research needs to focus on this problem and experimental protocols need to be developed in parallel, mainly in large animal models.

To indirectly increase the organ pool and improve the results of transplantation, tolerance induction still represents the "holy grail" to be reached and in this context,

large animal models need to be continuously studied. These models however, will be used only to confirm important and reproducible data found in mice models, especially when new mechanisms of tolerance are evidenced. In this context, the pig model still remains an appropriate one, because new pig reagents exist and the knowledge of the swine immune system is improving. In fact, there is no need to perpetuate more and more experiments that study very minor innovations, and large animal models must be reserved to confirm innovative approaches or new therapies. In this context, the combination of bone marrow transplantation and solid organ transplantation might represent the future of tolerance induction and thus, pig, dog, or primate models will still be used in the field, as SLA and DLA are well defined and we may be able to characterize the MHC in primates. In the field of tolerance, however, most of the progress will likely be made directly in human medicine, as so much has been already studied in animal models, that it is of overwhelming importance to study carefully the situation in clinical transplantation to solve the problems that are specific to human transplantation.

Similarly, a great deal of experimental work remains to be done in allotransplantation to transplant hypersensitized patients, who represent a significant percentage of cases. In this context, specific animal models must be developed and studied. Large animal models especially need to be developed to find new therapies that could be valuable for such patients. The comparability of the problem with xenotransplantation renders this question more up to date. The pig model seems to be appropriate for such studies and there is no need to use NHPs to be closer to the human situation, as pre-sensitization will provide in pigs, as in humans, an anti-MHC antibody response, and pig SLA is well enough defined to compare the results obtained with humans.

Xenotransplantation remains an important field of research for increasing the organ pool. Pigs have established a strong position as a large animal model in this area of research, as they appear to be the only possible organ and cell donors for humans. The availability of genetically modified pigs represents the second major point for developing the pig-to-primate model. GalT-KO pigs represent the first line, which should be improved by additional human genes and complementary regulatory proteins such as CD55 should be added to the GalT-KO background. In addition, pigs should be modified to protect against coagulopathy and transgenic pigs expressing anticoagulant fusion proteins, based on tissue factor pathway inhibitor (TFPI-Tg and hirudin (Hir-Tg)) will also be produced on the GalT-KO/CD55 background.

Finally, significant progress needs to be made with cellular therapy and probably needs to include xenogeneic cellular therapy, which could provide interesting results in the near future. These models also need to be studied in large animal models before they are considered for new strategies in human therapies. In this field, xenotransplantation of pig islets cells could be the first application in humans.

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In conclusion, the use of animal models remains crucial to progress in transplantation medicine. The guidelines should reserve a place for research in rodent models to discover new genes and new biological pathways by using all the tools such as transgenic and knock-out animals. Once new therapies, new molecules, or new pathways have been evidenced in rodents, the question of their confirmation in large animal models arises. The choice between pig and NHP models will be adapted to the questions, to knowledge of the model and to the possibility of monitoring the results.

In this context, funding from communities such as the European Community are certainly not easy to obtain and are very competitive, but they are important since they mandate setting up networks that will control the experiments to be done with animals and as such avoid the recurrence of similar experiments in many laboratories. Today, there are two such networks in transplantation, one for tolerance induction in humans (RISET) and one in xenotransplantation (XENOME).

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Key Words: Animal model, Xenotransplantation, Allotransplantation, Tolerance cellular transplantation., Review

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